Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 613-618

5(α-BROMOACETYL)-2'-DEOXYURIDINE 5'-PHOSPHATE: A MECHANISM BASED AFFINITY LABEL FOR THYMIDYLATE SYNTHETASE

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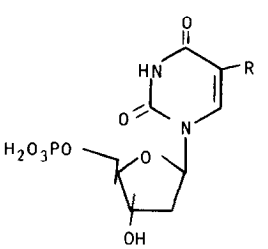
Received January 29,1979

SUMMARY

5(α-Bromoacetyl)-2'-deoxyuridine 5'-phosphate is an active site-directed irreversible inhibitor of thymidylate synthetase from Lactobacillus casei. The reversible inhibition (K_I 4uM) is competitive with substrate and on incubation the reversible enzyme-inhibitor complex is converted to the irreversible complex with a first order rate constant (k_2) of 0.15 min^{-1}.

INTRODUCTION

Thymidylate synthetase (E.C. 2.1.1.45) catalyzes the conversion of 2'-deoxyuridine 5'-phosphate (dUMP) to thymidine 5'-phosphate (dTMP). Inhibition of this process is a useful clinical method for controlling cancer growth and viral infections (1,2). Recently, two derivatives of the substrate have been characterized as mechanism based affinity labelling reagents for this enzyme. 5'-Iodoacetamidomethyl-(3) and 5-nitro-2'-deoxyuridine-5'-phosphate(4,5) are competitive inhibitors that inactivate the enzyme by conversion of the reversible enzyme-inhibitor complex to a covalently bound irreversible complex. The title compound (1) was designed and synthesized as a mechanism based affinity labelling reagent for thymidylate synthetase.



1 R = $COCH_2Br$

2 R = $CH_2NHCOCH_2I$

3 R = NO_2

0006-291X/79/060613-06$01.00/0

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EXPERIMENTAL

Thymidylate synthetase purified from methotrexate resistant Lactobacillus casei was purchased from the New England Enzyme Center, Tufts University, at a specific activity of 1.03 μmole of TMP formed per min per mg of protein using the radioisotope assay. The enzyme was activated by dialysis for 4 days at 4° against 0.1 M potassium phosphate (pH 6.8) containing 50 mM mercaptoethanol. The substrate 5 [^{3}H]-2'-deoxyuridine 5'-phosphate at a specific activity above 15 Ci/mmole was purchased from Moravek Biochemicals, Industry, California, and diluted with cold substrate purchased from Sigma Chemical Co., St. Louis, to give a specific activity of 500 uCi/μmole. The cofactor, dl-tetrahydrofolic acid, was also purchased from Sigma Chemical Co. The synthesis of the inhibitor 5(α-bromoacetyl)-2'-deoxyuridine 5'-phosphate was by bromination of 5-acetyl-2'-deoxyuridine and the resulting nucleoside was phosphorylated to give 1 which was characterized by CHN analysis and spectral data.

Enzyme Assay

The enzyme was assayed by modification of the radioisotope assays described by Roberts (6) and Lomax and Greenberg (7). The solution, 0.1 mL, contained 25 mM mercaptoethanol, 0.22 mM dl-tetrahydrofolic acid, 6.75 mM formaldehyde, 5 mM sodium bicarbonate, 3 mM magnesium chloride, 0.12 mM EDTA, 6 mM tris-acetate buffer pH 6.8, 5 μL of the diluted enzyme solution, substrate and when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 5-[^{3}H]-2'-deoxyuridine 5'-phosphate was used at a specific activity of 500 μCi/μmole. The assays were started by the addition of the enzyme to the complete mixture then incubated at 30°. Incubation was stopped at thirty seconds by the addition of 50 μL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added, the solution vortexed, and allowed to stand 15 min. The suspension was filtered through a glass wool plugged Pasteur pipette and 0.1 ml of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts.

Preincubation Studies

The enzyme ($\sim 5 \times 10^{-8}$ M) was preincubated at 30° in 50 μL of solution containing 5 mM 2-mercaptoethanol, 6 mM magnesium chloride, 0.24 mM EDTA, 12 mM tris-acetate buffer pH 6.8 and varying concentrations of inhibitor. After incubation for the indicated time period, the assay for remaining active enzyme was started by the addition of 50 μL of a solution containing buffer and other components of the assay to give the same concentrations as noted in the enzyme assay. A high substrate concentration (40 μM) was used in these assays to afford reasonably high velocity and to competitively reduce the enzyme inactivation by the inhibitor during the assay. The assay was run for 30 sec and treated as described in the enzyme assay section. Inactivation of the enzyme was measured by comparing the velocity at time zero to that at the indicated incubation times. Under the conditions of the assay the uninhibited enzyme retained 95% of the initial activity after twenty minutes of incubation.

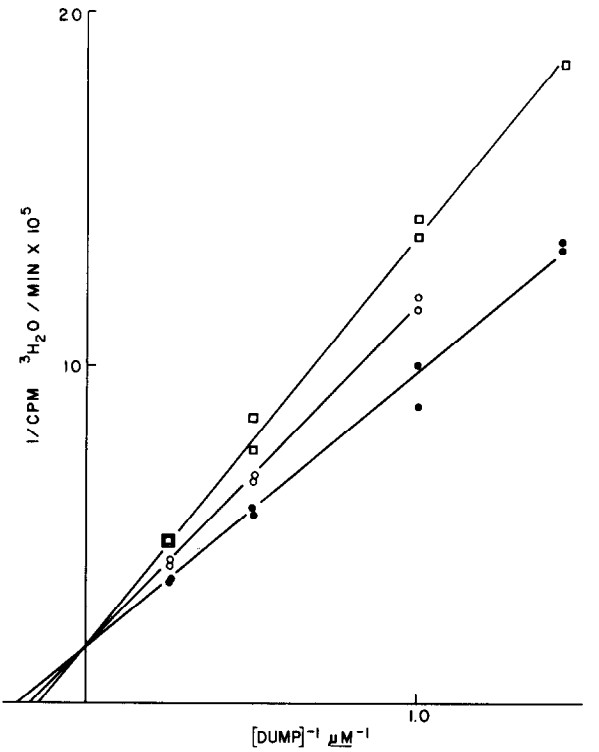


Figure 1. Double reciprocal plot of the cpm/min of ^3H_2O released by thymidylate synthetase catalysis vs concentration in uM of the substrate 5-[^{3}H]-2'-deoxyuridine 5'-phosphate (dUMP) in the presence of varying concentrations of the inhibitor, 5-(α-bromoacetyl)-2'-deoxyuridine 5'-phosphate; I=0 ●; I=1 uM o; I=2.0 uM □.

RESULTS AND DISCUSSION

Thymidylate synthetase purified from methotrexate resistant Lactobacillus casei was assayed in a thirty second radiolabelled assay to give (Figure 1) a K_m for the substrate of 5.0 uM. Compound 1 inhibited the enzyme and, from the double reciprocal plot in Figure 1, the inhibition was competitive with substrate; a K_I of 4.1 uM was calculated.

Preincubation of the mercaptoethanol activated enzyme with the inhibitor showed time and concentration dependent loss of enzyme activity. The linear plot of the log of the remaining enzyme activity vs time followed pseudo first order kinetics

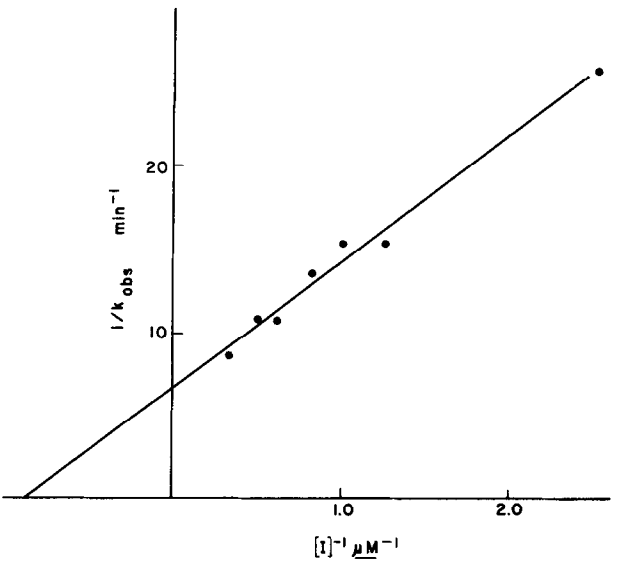


Figure 2. Double reciprocal plot of the observed rate (k_{obs}) of inactivation of thymidylate synthetase vs the concentration in uM of the inhibitor, 5(α-bromoacetyl)-2'-deoxyuridine 5'-phosphate.

wherein the slope of the lines is $-k_{obs}$. According to the analysis of Kitz and Wilson (8) the derived equation for the process can be plotted to obtain the first order rate constant for the conversion of the reversible enzyme-inhibitor (EI) complex to the irreversible complex (EI*). A double reciprocal plot (Figure 2) of the observed pseudo first order rate vs the inhibitor concentration in the absence of substrate gives a k_2 of 0.15 min^{-1} as calculated from the $1/k_{obs}$ intercept.

$$E + I \underset{K_I}{\rightleftharpoons} EI \xrightarrow{K_2} EI^*$$

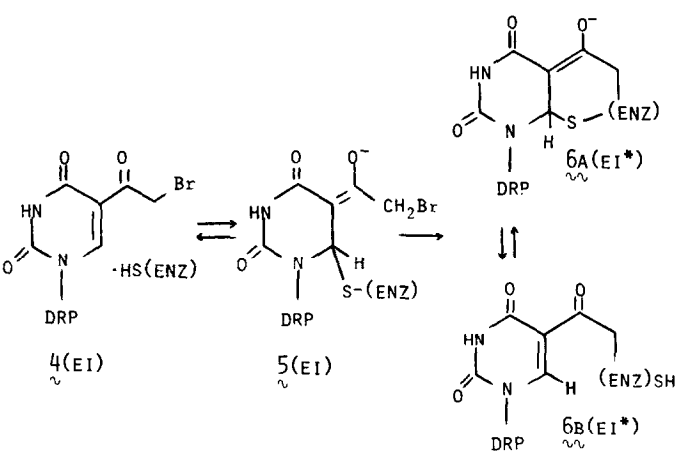
$$E + S \underset{K_m}{\rightleftharpoons} ES$$

$$\frac{1}{K_{OBS}} = \frac{K_I}{K_2}\frac{1}{[I]} + \frac{1}{K_2}$$

In comparison to the 5-iodoacetamido derivative (2), compound 1 has over ten times the affinity of 2 for thymidylate synthetase, however, the rate of inactivation by 1 is only four times greater than that of 2 which is 0.057/min for k_2. These differences could be a result of species enzymic changes since the constants for 2 were derived from enzyme purified from Ehrlich ascites tumor.

On the other hand, compound 3, the 5-nitro derivative, has one hundred times greater affinity ($K_I \sim 0.03$ uM) for this enzyme than 1. Furthermore, the rate of inactivation by 3, k_2, greatly exceeds the rate of the first step leading to the reversible EI complex. Accordingly, the second order rate constant for 3 (k_2) in the equation $-dE/dt = k_2[E][I]$ is 6.6×10^5 $\ell mole^{-1}$ sec^{-1} (9).

Although the mechanism of inactivation is uncertain at this time, details of the catalytic enzyme mechanism (10,11) provide a reasonable route. The presence of strong electron withdrawing groups (such as a carbonyl) at carbon-5 of the substrate should enhance the Michael addition of the active site cysteine to carbon-6 of the inhibitor (4→5, Scheme 1).



Scheme 1 Proposed Mechanism of Inactivation of Thymidylate Synthetase

A second nucleophile is proposed in the catalytic process to abstract the carbon-5 proton in the intermediate ternary complex of enzyme-substrate-cofactor to give elimination of the enzyme and the products dTMP and 7,8-dihydrofolic acid. The chemically reactive α-halocarbonyl of 1 in the reversible complex 5 could alkylate this second nucleophile at the active site to give the irreversible enzyme-inhibitor complex formulated as 6a or 6b.

ACKNOWLEDGEMENTS

This work was supported by a grant (CA 7522) from the National Cancer Institute of the National Institutes of Health.

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